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***Roseobacter gallaeciensis* sp. nov., a new marine bacterium isolated from rearings and collectors of the scallop *Pecten maximus*.**

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Abstract

Four bacterial strains were isolated from larval cultures and collectors of the scallop *Pecten maximus*. They showed a high level of intragroup genomic relatedness (84–95%) as determined by DNA–DNA hybridization. The cells were Gram-negative, strictly aerobic, motile, ovoid rods. They grew at temperatures from 15 to 37 °C and from pH 7.0 to 10, but did not grow in the absence of NaCl and required growth factors. They had the ability to use a wide variety of compounds as sole carbon source: D-mannose, D-galactose, D-fructose, D-glucose, D-xylose, melibiose, trehalose, maltose, cellobiose, sucrose, mesoerythritol, D-mannitol, glycerol, D-sorbitol, meso-inositol, succinate, propionate, butyrate, c-aminobutyrate, DL-hydroxybutyrate, 2-ketoglutarate, pyruvate, fumarate, glycine, L- α -alanine, β -alanine, L-glutamate, L-arginine, L-lysine, L-ornithine and L-proline. They exhibited oxidase and catalase activities but no denitrification activity. The isolates did not contain bacteriochlorophyll a. The GMC content ranged from 57.6 to 58 mol%. Phylogenetic analyses of the 16S rRNA sequence revealed that these isolates belong to the genus *Roseobacter*. On the basis of quantitative hybridization data, it is proposed that these isolates should be placed in a new species, *Roseobacter gallaeciensis*. The type strain is *Roseobacter gallaeciensis* BS107^T (vCIP 105210^T).

Keywords: *Roseobacter gallaeciensis* sp. nov., marine bacterium, scallop, *Pecten maximus*

INTRODUCTION

The genus *Roseobacter* was created by Shiba (15) to include two bacterial species, *Roseobacter litoralis* and *Roseobacter denitrificans*, which were obtained from the surface of green seaweeds. Recently, Lafay et al. (9) described a new species, *Roseobacter aquicola*, which includes three bacterial strains found in cultures of the toxin-producing dinoflagellate *Prorocentrum lima*.

Four bacterial strains (BS107, BSc1, BSc4, BSc20) isolated from larval cultures and collectors of the scallop *Pecten maximus* were found to exhibit an antibacterial effect against a broad spectrum of bacteria, especially *Vibrio* spp. Moreover, extracts of isolate BS107 added to scallop cultures produced a probiotic effect on larvae (12). Based on a phenotypic examination as well as phylogenetic analyses of the EMBL accession number for the sequence reported in this paper is Y13244. small-subunit rRNA, the latter performed with the most representative strain (BS107), we have shown that these bacteria should be included in the genus *Roseobacter*. Data obtained from DNA–DNA hybridization suggest that these isolates are affiliated to a new species for which we propose the name *Roseobacter*

gallaeciensis. The type strain is *Roseobacter gallaeciensis* BS107T (= CIP 105210T, Collection de l'Institut Pasteur, Paris, France).

METHODS

Bacterial strains and growth conditions. Strain BS107T was found in seawater from larval cultures of the scallop *Pecten maximus* (Instituto Oceanografico, A Coruña, Spain). Strains BSc1, BSc4 and BSc20 were isolated from the surface of collectors used to allow scallop larval settlement (Instituto Oceanografico). All strains were selected on marine agar 2216 (MA; Difco). Subcultures of *Roseobacter aquicola*, *Roseobacter denitrificans* and *Roseobacter litoralis* derived from ATCC strains 51440T, 33942T and 49566T, respectively, were kindly provided by IFREMER and used as reference strains.

The strains were stored at -80 °C in marine broth 2216 (MB; Difco) supplemented with 20% (v/v) glycerol. Cultures were grown at 23 °C, either in MB or in artificial Shioi's marine medium (SMM) as adapted by Shiba (16). Growth requirements were determined by inoculating exponential- phase cells in artificial seawater (ASW; solution of Sigma sea salts) in three combinations, each lacking one of the following compounds: NH₄Cl (1g l⁻¹), glucose (0 -1 g l⁻¹) or casein hydrolysate (10 mg l⁻¹). One control contained none of these compounds and another contained all of them. Requirements for biotin, thiamin, nicotinic acid and sodium pantothenate were determined by the method used by Shiba (15). The strains did not grow on thiosulfate/citrate/bile salts agar.

Phenotypic tests. Cell shape and flagella were observed by phase-contrast microscopy (Nikon) after specific staining for flagella (8). Cell size and a more detailed morphology were determined by transmission electron microscopy, for which cells were negatively stained with 1% (w/v) uranyl acetate, using Formvar (300 mesh Cu grid ; Oxford Instruments) as described by Paillard (11).

Routine tests (Gram staining, oxidase, catalase, amylase, β -galactosidase, gelatinase, tweenase, DNase and urease activity tests, and reduction of nitrate) were performed with exponential-phase cells as described by Smibert & Krieg (17). Motility was examined by the hanging drop method with 24 h cultures grown in MB. Pigment production (colour of colonies and diffusible pigment production) was observed on MA, MA supplemented with 0 - 5% starch and SSM after 7 d incubation.

Metabolism of glucose was determined by a modification of the oxidation/fermentation method (MOF) of Hugh & Leifson (10). Metabolism of citrate was tested by using Simmon's citrate medium (Difco) (17). Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activities were determined by using techniques described by Baumann & Baumann (1) for marine bacteria. We also observed the effects of pH (4 to 10), temperature (4, 15, 20, 37 and 40 °C) and NaCl concentration (0, 0.1, 0.25, 0.5, 0.7, 1, 1.5 and 2 M) on growth on MA. Results were recorded after 4 d incubation.

Utilization of different carbon sources was tested by using ASW with casein hydrolysate (10 mg l⁻¹) and the following substrates, each at a concentration of 0.1%: d-mannose, d-galactose, d-fructose, d-glucose, d-xylose, l-arabinose, l-rhamnose, trehalose, maltose, cellobiose, melibiose, sucrose, meso-erythritol, d-mannitol, glycerol, d-sorbitol, mesoinositol, glucosamine, salicin, d-gluconate, succinate, citrate, galacturonate, acetate, propionate, butyrate, c-aminobutyrate, dl-hydroxybutyrate, isovalerate, 2-ketoglutarate, pyruvate, fumarate, l-tyrosine, glycine, l- α -alanine, β -alanine, dl-serine, l-leucine, l-valine, l-aspartate, l-glutamate, l-arginine, l-lysine, l-ornithine, l-histidine, l-proline and betaine. Growth in the presence of different carbon sources using the organic acid medium described by Shiba (15)

was not studied as we obtained the same results when *Roseobacter aquicola* FF3T (9) was inoculated in this medium and in the medium described above (data not shown).

Susceptibility to different inhibitors was tested on MA by using the standard antibiogram method (2) and the following antibiotics : penicillin G (10 U), chloramphenicol (30 µg), kanamycin (30 U), erythromycin (15 U), oxolinic acid (10 µg), gentamicin (10 U), thrimethoprim (1.25 µg)- sulfamethoxazole (23.75 µg), furan (300 µg), streptomycin (100 U) and vibriostatic agent O/129 (2,4-diamino-6,7- diisopropyl pteridine) (150 µg) (all from Sanofi Diagnostics). Production of bacteriochlorophyll a was determined by spectrophotometric analysis. Cells grown in 200 ml SSM, collected by centrifugation as described by Lafay et al. (9) and the resulting pellet was extracted with 100 ml methanol at 4 °C. The methanolic extract was concentrated under vacuum at 35 °C in the dark to a volume of 1 ml. The presence of pigment was determined by visible spectrophotometry (350 -900 nm).

DNA base composition. Isolation of genomic DNA was carried out by standard methods (14). The G-C content was calculated according to the thermal denaturation midpoint (T_m) method (4).

DNA amplification. A modification of the method of Sritharan & Barker (18) was followed to prepare genomic DNA for PCR. Bacteria grown in MB were centrifuged at 3000 g at 4 °C for 10 min. The pellet obtained was resuspended in 200 µl lysis mixture (10 mM Tris/HCl, pH 8 -0, 1 mM EDTA, 1% Triton X-100) and boiled for 5 min. After a single chloroform extraction, 5 µl supernatant was used to amplify the 16S rRNA gene with Taq DNA polymerase (Stratagene) and two primers, which corresponded to positions 8-28 and 1493-1509 in the *Escherichia coli* smallsubunit rRNA sequence. The initial denaturation step consisted of heating the reaction mixture at 95 °C for 180s. Immediately, the genomic DNA was exposed to 10 cycles of amplification consisting of denaturation at 95 °C for 90 s, annealing at 50 °C for 90 s and extension at 72 °C for 180 s, followed by 10 cycles of 95 °C for 90 s, 50 °C for 90 s and 72 °C for 4 min, and 20 cycles of 95 °C for 90 s, 50 °C for 90 s and 72 °C for 5 min. A final extension step was performed at 72 °C for 10 min. Two nanograms of the PCR products was used for reamplification, performed under the same conditions except for the utilization of Pfu DNA polymerase (Stratagene) instead of Taq DNA polymerase. Pfu DNA polymerase forms blunt-ended DNA which is easier to clone within a standard plasmid.

Cloning and sequencing. The PCR products were analysed on a 1% agarose gel which included a molecular mass standard (phage λ digested with PstI) for the quantification of the products. The fragment required was purified by using GeneClean (Bio101) and cloned into pBluescript KS(+) using PCR Script (Stratagene) (14). After cell transformation, 10 positive clones were grown individually in SOB/ ampicillin medium (14). Plasmid DNA was extracted immediately (14), digested with SacI and HindIII, and electrophoresed in 1% agarose. Both strands of the DNA corresponding to the small fragments obtained by electrophoresis were denatured and sequenced by the dideoxy chain-termination method using the USB Sequenase Quick-Denature Plasmid Sequencing Kit. Reverse and universal primers homologous to the nucleotide sequences of pBluescript adjacent to the inserted DNA were used for sequencing the gene, as well as primers S10 and S12 which correspond to positions 906-925 and 1099-1114, respectively, in the *E. coli* sequence. No significant differences were found in the sequences of the clones.

Phylogenetic analyses. Phylogenetic data were obtained by aligning different sequences and other phylogenetic procedures. All analyses were done using computer programs developed

by Galtier et al. (6). Only nucleotide positions which aligned without ambiguities with the different 16S rRNA sequences were used for phylogenetic analyses. Three phylogenetic methods were employed, all included within the Phylo-Win program package (6). (i) Neighbour-joining algorithm. A neighbour-joining algorithm similar to that developed by Saitou & Nei (13) was used. (ii) Maximum-parsimony. The Syst Zol 20:406 algorithm from the phylip package (5) was used. Bootstrap analysis (100 replications) was performed to check each topology for robustness. (iii) Maximum-likelihood. The fdnaml program derived from the dnaml program (5) and rewritten by G. J. Olsen (University of Illinois, Urbana, IL, USA) was used. All analyses were performed using the global option (F, Y and G options). All trees obtained with these phylogenetic methods were plotted by using a Macintosh computer and the program njplot developed by M. Gouy (URA 243 CNRS, Université Claude Bernard, Villeurbanne, France) which allowed a formal tree representation (Newick's format) to be transformed into ClarisDraw drawings. Only topologies that were found to be similar by all three methods were retained as 'true trees'.

Nucleotide sequence accession numbers. Sequences used in this study correspond to the following accession numbers: *Roseobacter algicola* ATCC 51440T, X78315; *Roseobacter denitrificans* OCH 114T, M59063; *Roseobacter litoralis* ATCC 49566T, X78312; *Rhodobacter sphaeroides* IFO 12203, D16425; *Rhodobacter capsulatus* ATCC 11166, D16428.

Quantitative DNA–DNA hybridization. Genomic DNA from the new isolates and the reference strains, *Roseobacter algicola*, *Roseobacter denitrificans* and *Roseobacter litoralis*, was extracted following standard methods (14). Strain BS107T and *Roseobacter algicola* probes were labelled by nick-translation (14). Hybridization was carried out at 72 °C and the S1 nuclease method, with adsorption of S1-resistant DNA onto DE81 filters (Whatman), was followed to calculate the percentage reassociation (3, 7).

RESULTS

Cell shape, motility and Gram staining Cells of all four isolates were Gram-negative and ovoid-rod-shaped (0.7-1 µm wide, 1.7-2.5 µm long). They were motile by means of polar flagella. Several trials failed to determine the number of flagella because electron microscopy observations revealed only a few flagellated cells and many that loose their flagella (data not shown). However, a polar flagellum was observed by phase-contrast microscopy.

Culture and growth characteristics On MA at 23 °C, young colonies were circular (diam. ~0.5 mm), smooth, convex and brownish with regular edges. After 7 d incubation, colonies were 2 mm in diameter and brown with irregular edges. They produced diffusible pigment. The same results were obtained on MA supplemented with 0.5% starch. The isolates were not capable of utilizing ammonium as nitrogen source. They grew at temperatures between 15 and 37 °C, optimal growth occurring at 23-27 °C. Growth was observed at pH 7.0-10.0 and the optimum pH was 7.0; no growth was observed at pH 4.0. They grew at salt concentrations ranging from 0.1 to 2M, the optimum salt concentration being 0.2 M, and required thiamin.

Biochemical and physiological characteristics Phenotypic characteristics that differentiated *Roseobacter gallaeciensis* from other *Roseobacter* spp. are shown in Table 1. The four isolates exhibited catalase and oxidase activities. Amylase, gelatinase, b-galactosidase, tweenase, DNase, urease, arginine dihydrolase, lysine decarboxylase and ornithine

decarboxylase activities were not detected. The isolates were not able to reduce nitrate to nitrite. They exhibited oxidative metabolism of glucose, but did not metabolize citrate. The four isolates were able to use the following substrates as sole carbon and energy source: d-mannose, d-galactose, d-fructose, d-glucose, d-xylose, melibiose, trehalose, maltose, cellobiose, sucrose, meso-erythritol, d-mannitol, glycerol, d-sorbitol, meso-inositol, succinate, propionate, butyrate, c-aminobutyrate, dl-hydroxybutyrate, 2-ketoglutarate, pyruvate, fumarate, glycine, l-a-alanine, b-alanine, l-glutamate, l-lysine, l-arginine, l-ornithine and l-proline. Other compounds were used variably, for example acetate and leucine were only used by BSc1 and BS107T, respectively, and arginine was used by all isolates except BSc4. Bacteriochlorophyll a was not detected in the methanolic extracts of the isolates (Fig. 1). The peak observed did not correspond to bacteriochlorophyll a (16).

Table 1. Characteristics that differentiate *Roseobacter gallaeciensis* from *Roseobacter litoralis*, *Roseobacter denitrificans* and *Roseobacter algicola*

+, Positive reaction; —, negative reaction.

Characteristic	<i>Roseobacter litoralis</i> ATCC 49566 ^T	<i>Roseobacter denitrificans</i> ATCC 33942 ^T	<i>Roseobacter algicola</i> ATCC 51440 ^T	<i>Roseobacter gallaeciensis</i> BS107 ^T
Morphology	Rods or ovoid rods	Rods or ovoid rods	Ovoid rods	Ovoid rods
Growth at 4 °C	+	+	—	—
Growth at 37 °C	—	—	+	+
Hydrolysis of:				
Starch	—	—	+	—
Tween 80	+	+	—	—
Gelatin	+	+	+	—
Requirement for nicotinic acid	+	+	—	—
Utilization of carbon sources:				
Maltose	—	—	+	+
Sucrose	—	—	+	+
Trehalose	—	—	+	+
Citrate	+	+	+	—
Butyrate	—	—	—	+
2-Ketoglutarate	+	—	+	+
Nitrate reduced to nitrite	—	+	—	—
Susceptibility to penicillin G (10 U)	+	+	+	—
Production of bacteriochlorophyll a	+	+	—	—
Non-diffusible pigment	Pink to red	Pink to red	Beige	Brownish
Diffusible pigment	—	—	None or brown	Brown
G + C content (mol %)	56–58.8	59.6	60	57.6–58.0

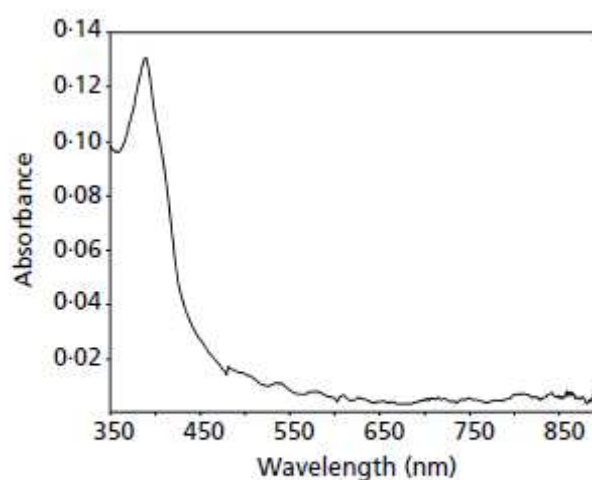
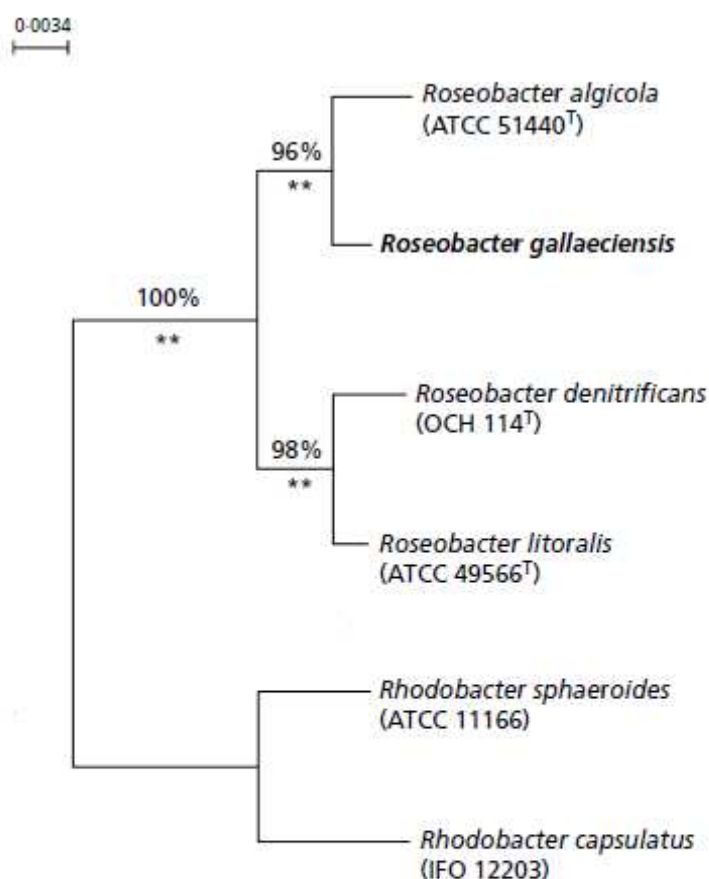


Fig. 1. Absorption spectrum of a methanolic extract of strain BS107^T.

Susceptibility to inhibitors All four isolates were sensitive to kanamycin, erythromycin, oxolinic acid, gentamicin, streptomycin and vibriostatic agent O/129. BS107^T was the only isolate sensitive to chloramphenicol.

Phylogenetic analyses of small-subunit rRNA Phylogenetic analyses, including different representatives of the Proteobacteria (data not shown), indicated that BS107^T was clustered within the α subclass of the Proteobacteria. The position of this bacterium within the α subclass was investigated. The three methods described in Methods were congruent and showed that the new bacterium clustered with the monophyletic unit of *Roseobacter* spp. (Fig. 2). This position was assured by a value of 100% replication of bootstrap (maximum parsimony and maximum likelihood). The most parsimonious tree was found with maximum-parsimony analysis. All branches were well supported in maximum-likelihood analysis. This result was confirmed by the high level of similarity of the small-subunit rRNA sequences found between the unidentified bacterium and strains of *Roseobacter algicola* (98.8%), *Roseobacter litoralis* (97.8%) and *Roseobacter denitrificans* (97.4%). These values were obtained by the distance matrix of the neighbour-joining algorithm.



G-C content and DNA-DNA hybridization The new isolates had DNA G-C contents of 57.6-58 mol%. In consideration of the phylogenetic tree based on small-subunit rRNA sequences and the high levels of similarity (more than 97%) found between BS107^T and *Roseobacter* spp., quantitative DNA-DNA hybridization assays were performed. We also carried out DNA-DNA hybridization assays between the new isolates to obtain detailed information about their genetic relationship. Results obtained revealed a very low level of homology with *Roseobacter algicola* (2.4%), *Roseobacter denitrificans* (2%) and *Roseobacter litoralis* (3%) but the levels of genomic DNA hybridization between the new isolates were more than 70% (Table 2).

Table 2. G+C contents and levels of genomic DNA relatedness between the new isolates and reference strains

Strain	G+C content (mol %)	Hybridization (%) with labelled DNA from:	
		BS107 ^T (= CIP 105210 ^T)	<i>Roseobacter algicola</i> (ATCC 51440 ^T)
BS107 ^T (= CIP 105210 ^T)	58.0	100	4
BSc1	57.6	84	2
BSc4	58.0	95	3
BSc20	58.0	93	3
<i>Roseobacter algicola</i> (ATCC 51440 ^T)	60.0	3	100
<i>Roseobacter denitrificans</i> (ATCC 33942 ^T)	59.6	2	1
<i>Roseobacter litoralis</i> (ATCC 49566 ^T)	58.8	3	2

DISCUSSION

The phylogenetic analyses have demonstrated the affiliation of isolate BS107^T to species of the genus *Roseobacter* of the a subclass of the Proteobacteria, particularly *Roseobacter algicola*. This result was confirmed by the solid monophyletic group formed by *Roseobacter* spp. (100% replication of bootstrap). These data, as well as phenotypic characteristics of the four isolates, are sufficiently conclusive to allow assignment of these isolates to the genus *Roseobacter*. The low levels of DNA reassociation found between the new isolates and the three known species of *Roseobacter*, *Roseobacter algicola*, *Roseobacter denitrificans* and *Roseobacter litoralis*, as well as the high level of DNA homology observed between the new isolates, indicate that the new isolates can be grouped in a new species of the genus *Roseobacter* for which we propose the name *Roseobacter gallaeciensis*. Description of *Roseobacter gallaeciensis* sp. nov. *Roseobacter gallaeciensis* [gal.lae.ci.en«sis. L. adj. of/from Galicia (L. name Gallaecia), North-West region of Spain]. Cells are Gram-negative ovoid rods (length 1.7-2.5 µm; width 0.7-1 µm) and motile by means of polar flagella. Colonies on salt-containing agar medium are brownish in young cultures and brown after 72-96 h incubation. Cells grow at temperatures ranging from 15 to 37 °C. Optimal growth occurs at 23-27 °C and at a pH around 7.0. Cells grow in the presence of 0.1-2 M NaCl, the optimum concentration being 0.2 M, require thiamin and demonstrate nonfermentative metabolism. They exhibit oxidase and catalase activities but no denitrification activity. d-Mannose, d-galactose, d-fructose, d-glucose, d-xylose, melibiose, trehalose, maltose, cellobiose, sucrose, meso-erythritol, d-mannitol, glycerol, d-sorbitol, meso-inositol, succinate, propionate, butyrate, c-aminobutyrate, dl-hydroxybutyrate, 2-ketoglutarate, pyruvate, fumarate, glycine, l-a-alanine, b-alanine, l-glutamate, l-arginine, l-lysine, l-ornithine and l-proline are used. Cells are resistant to penicillin G, thrimethoprim - sulfamethoxazole and furan, and are susceptible to kanamycin and vibriostatic agent O/129. Cells do not contain bacteriochlorophyll a. The G-C content ranges from 57.6 to 58 mol%. Phylogenetic analysis places the isolates in a distinct monophyletic group of the a subclass of the Proteobacteria containing *Roseobacter*

spp. Habitats are seawater and marine environments. Type strain is BS107^T (=CIP 105210^T) isolated from seawater of larval cultures of the scallop *Pecten maximus* in A Coruña, Galicia, Spain. G-C content of type strain is 58 mol%.

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